



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Diagnostic evaluation of assays for detection of antibodies against porcine epidemic diarrhea virus (PEDV) in pigs exposed to different PEDV strains

Citation for published version:

Gerber, PF, Lelli, D, Zhang, J, Strandbygaard, B, Moreno, A, Lavazza, A, Perulli, S, Bøtner, A, Comtet, L, Roche, M, Pourquier, P, Wang, C & Opriessnig, T 2016, 'Diagnostic evaluation of assays for detection of antibodies against porcine epidemic diarrhea virus (PEDV) in pigs exposed to different PEDV strains' Preventive Veterinary Medicine, vol. 135, pp. 87-94. DOI: 10.1016/j.prevetmed.2016.11.005

Digital Object Identifier (DOI):

[10.1016/j.prevetmed.2016.11.005](https://doi.org/10.1016/j.prevetmed.2016.11.005)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Preventive Veterinary Medicine

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



Accepted Manuscript

Title: Diagnostic evaluation of assays for detection of antibodies against porcine epidemic diarrhea virus (PEDV) in pigs exposed to different PEDV strains

Author: Priscilla F. Gerber Davide Lelli Jianqiang Zhang Bertel Strandbygaard Ana Moreno Antonio Lavazza Simona Perulli Anette Bøtner Loic Comtet Mickael Roche Philippe Pourquier Chong Wang Tanja Opriessnig



PII: S0167-5877(16)30535-9
DOI: <http://dx.doi.org/doi:10.1016/j.prevetmed.2016.11.005>
Reference: PREVET 4136

To appear in: *PREVET*

Received date: 10-5-2016
Revised date: 31-10-2016
Accepted date: 6-11-2016

Please cite this article as: Gerber, Priscilla F., Lelli, Davide, Zhang, Jianqiang, Strandbygaard, Bertel, Moreno, Ana, Lavazza, Antonio, Perulli, Simona, Bøtner, Anette, Comtet, Loic, Roche, Mickael, Pourquier, Philippe, Wang, Chong, Opriessnig, Tanja, Diagnostic evaluation of assays for detection of antibodies against porcine epidemic diarrhea virus (PEDV) in pigs exposed to different PEDV strains. *Preventive Veterinary Medicine* <http://dx.doi.org/10.1016/j.prevetmed.2016.11.005>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Diagnostic evaluation of assays for detection of antibodies against porcine epidemic diarrhea virus (PEDV) in pigs exposed to different PEDV strains

Priscilla F Gerber ¹, Davide Lelli ², Jianqiang Zhang ³, Bertel Strandbygaard ⁴, Ana Moreno ², Antonio Lavazza ², Simona Perulli ², Anette Bøtner ⁴, Loic Comtet ⁵, Mickael Roche ⁵, Philippe Pourquier ⁵, Chong Wang ³, Tanja Opriessnig ^{1,3*}

¹ *The Roslin Institute and The Royal (Dick) School of Veterinary Studies, University of Edinburgh, Midlothian, United Kingdom*

² *Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini", Brescia, Italy*

³ *Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, Iowa, USA*

⁴ *DTU National Veterinary Institute, Kalvehave, Denmark*

⁵ *Innovative Diagnostics IDvet, Grabels, France*

**Corresponding author at: The Roslin Institute and The Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Midlothian, EH25 9RG, United Kingdom. Tel.: +44 (0)131 651 9177. E-mail address: Tanja.Opriessnig@roslin.ed.ac.uk (T. Opriessnig).*

Abstract

Porcine epidemic diarrhea virus (PEDV) has caused economic losses in the Americas, Asia and Europe in recent years. Reliable serological assays are essential for epidemiological studies and vaccine evaluation. The objective of this study was to compare the ability of five enzyme-linked immunosorbent assays (ELISAs) to detect antibodies against different PEDV strains in pig serum. A total of 732 serum samples from North American or European pigs were tested. Samples included experimental samples from pigs infected with classical (G1a PEDV) or variant genogroup 1 PEDV (G1b PEDV), pandemic genogroup 2 PEDV (G2b PEDV) or non-infected controls. Field samples from herds with confirmed or unknown PEDV exposure were also used. Three indirect ELISAs based on G2b antigens (ELISAs 1, 2 and 3), a competitive ELISA based on the G2b antigen (ELISA 4) and a competitive ELISA based on the G1a antigen (ELISA 5) were compared. Overall, the tests had a moderate agreement ($\kappa = 0.61$). G1a PEDV infected pigs were earliest detected by ELISA 3, G1b PEDV infected pigs were earliest detected by ELISAs 4 and 5 and the performance of all tests was similar for the G2b PEDV group. ELISA 1 showed the overall lowest detection on experimentally and field derived samples. Diagnostic sensitivity and specificity with a 95% probability interval were estimated to be 68.2% (62.1 – 74.4%) and 97.5% (95.2 – 99.0%) for ELISA 1, 73.7% (71.5 – 79.6%) and 98.4% (96.6 – 99.5%) for ELISA 2, 86.2% (81.1 – 90.6%) and 91.6% (87.7 – 94.8%) for ELISA 3, 78.3% (72.8 – 83.5%) and 99.7% (98.2 – 100%) for ELISA 4, and 93.5% (90.3 – 96.0%) and 91.2% (83.8 – 97.9%) for ELISA 5.

Differences in detection among assays seem to be more related to intrinsic factors of an assay than to the PEDV antigen used.

Words: 288/400

Keywords: Porcine epidemic diarrhea virus, ELISA, global comparison, diagnosis.

1. Introduction

Since the first observed porcine epidemic diarrhea virus (PEDV) outbreak in the UK in 1971, the virus has been reported in swine producing countries in Europe, Asia, and more recently, in the Americas and Caribbean (Song et al., 2015). The PEDV is highly contagious and causes enteric disease characterized by acute vomiting and diarrhea in pigs of all ages and often with up to 100% of mortality in suckling pigs in naïve breeding herds (Saif et al., 2012). The virus belongs to the family *Coronaviridae*, genus *Alphacoronavirus*, along with transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV) (Masters, 2006). Coronaviruses contain four major structural proteins, the spike (S) protein, the envelope (E) protein, the membrane (M) protein and the nucleocapsid (N) protein (Brian and Baric, 2005).

PEDV can be grouped into genogroups 1 (G1a and G1b) and 2 (G2a and G2b) strains based on amino acid differences in the N-terminal domain of the S gene (Huang et al., 2013; Lee, 2015). Most PEDV strains circulating in Europe and in Asia prior to 2010 belong to G1a, including the historical and vaccine strains, such as CV777 and DR-13 (Song and Park, 2012). Genogroup 1b strains, also known as S-INDEL strains, comprise new variant strains that contain genetic signatures of the classical G1a strains in their S gene and appear to have resulted from a recombination event between G1a and epidemic G2b viruses, also known as non-S-INDEL or pandemic strains (Lee, 2015). Genogroup 1b strains have been reported in Asia, the North America and in Europe (Lee, 2015). Since 2010, large-scale outbreaks with higher mortality in suckling piglets than previously described in Asia have been associated with PEDV G2a isolates, which are restricted to Asia, and G2b isolates (Song et al., 2015). In 2013, a PEDV G2b isolate similar to PEDV strains circulating in China since 2011, was introduced into a swine dense area in the Midwest of the US. Following introduction, G2b PEDV spread

rapidly across the US, further into Canada, and also into swine producing countries in Latin America (Song et al., 2015).

A number of methods have been used to demonstrate PEDV antibodies such as enzyme-linked immunosorbent assays (ELISAs) and immunofluorescence assays (IFAs). Available assays are either based on the whole virus (WV) (Hofmann and Wyler, 1990; Oh et al., 2005; Thomas et al., 2015), the N protein (Hou et al., 2007; Okda et al., 2015) or the S protein (Gerber et al., 2014a; Knuchel et al., 1992). The N protein, the most abundant viral protein expressed in PEDV infected cells, is highly conserved among PEDV genogroups (96-99.7% amino acid identity). The amino acid identity of the PEDV N protein is lower than 35% when compared to other porcine coronaviruses, however, it contains epitopes highly conserved among the family *Coronaviridae*, and cross reaction with certain TGEV strains have been reported (Lin et al., 2015). An S-protein based ELISA has been shown to be more specific and sensitive than an N-protein based ELISA (Knuchel et al., 1992), however, it has been suggested that the high heterogeneity of the S1 protein among PEDV isolates within and between genogroups (91-99.6% amino acid identity) could limit the sensitivity of S protein-based assays in the field (Lin et al., 2015).

The objective of the current study was to assess the diagnostic performance of two commercial and three *in-house* enzyme immunoassays based on PEDV WV antigen, N proteins or the S1 protein. To evaluate the assays, serum samples obtained from pigs in research facilities in the US or Denmark and commercial operations in Italy and the US without exposure to PEDV, pigs experimentally infected with G1a PEDV, G1b PEDV or G2b PEDV strains and field serum samples with confirmed or unknown PEDV exposure were used.

2. Material and Methods

2.1. Serum samples

A total of 708 serum samples from pigs with known or unknown PEDV exposure status were included in this study. A sample size of 160 samples was calculated based on a conservative value of 0.5 for both sensitivity and specificity to warrant a probability of more than 99% and to obtain a 95% confidence interval half-width less than or equal to 0.1 in the estimation of one sample proportion. After meeting the minimum requirement, the total number of samples included in this study was based on purposefully collecting samples in farms with known coronavirus status and on availability of convenience samples. The serum samples were obtained from pigs of different age groups: suckling (1 to 3-4 weeks of age), nursery (3-4 to 10 weeks of age), grow-finish (10 to 25 weeks of age) pigs and adult (> 25 weeks of age) pigs. Some of the samples were selected from random submissions to Veterinary Diagnostic Laboratories and an age was not available. These samples were classified as “unknown age”. None of the herds vaccinated pigs against PEDV at the time of sample collection.

2.1.1. Experimental samples with known PEDV exposure status

The experimental protocols were approved by the Danish Animal Experimentation Inspectorate and by the Iowa State Institutional Animal Care and Use Committee and the Institutional Biosafety Committee. The 22 PEDV negative serum samples were obtained from US (19 samples/19 pigs) and Danish (3 samples/3 pigs) uninfected control pigs. Sixty-four serum samples were obtained from Danish pigs infected with a G1a PEDV strain (15 samples/3 pigs), US pigs infected with a G1b-PEDV strain (24 samples/6 pigs), and US pigs infected with a G2b-PEDV strain (25 samples/13 pigs). Specifically, the Danish samples were obtained from 5-week-old pigs infected with PEDV G1a strain Br1/87 (Bridgen et al., 1993) at day post-inoculation (dpi) 10, 14, 17, 21 and 28 (Lohse et al., in press). In addition, another set of 3-week-old US samples was from pigs orally inoculated with PEDV G1b isolate USA/IL/2014/20697 P7 and samples were collected at dpi 7, 14, 21 and 28 (Chen et al., 2016b). Furthermore, one set of US samples was obtained from 3-week-old pigs infected with PEDV

G2b strain ISU13-19338E (GenBank n. KF650371) and collected at dpi 7, 14, and 21 (Gerber et al., 2014b; Opriessnig et al., 2014).

2.1.2. *Field samples with known PEDV exposure*

A total of 294 samples from US or Italian pigs from PEDV exposed or unexposed farms were used. The PEDV infection status of a given farm was determined based on demonstration of PEDV RNA in fecal samples by real-time reverse-transcriptase (RT)-PCR and presence of enteric signs. RT-PCRs were performed using a commercially available assay test (Tetracore, Rockville, US) at Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini" (IZSLER) or an *in-house* RT-PCR targeting the N gene at the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) (Chen et al., 2014).

Field samples with PEDV exposure. Twenty-five serum samples were collected from three Italian finishing pig farms 4-8 weeks after the start of a PEDV outbreak in 2006 (Martelli et al., 2008). Another 124 serum samples from nursery and grow-finish pigs were collected from 10 Italian farms between 2014 and 2015 4-8 weeks after the start of PEDV outbreaks in these farms. Sequencing analysis of the S1 gene from RT-PCR positive fecal samples identified a G1a PEDV strain in the 2006 outbreaks while G1b PEDV strains were identified in the 2014-2015 outbreaks (Boniotti et al., 2016).

Field samples without PEDV exposure. Farms were localized in areas with no previous history of enteric signs compatible with viral diarrhea, were PEDV RNA negative based on a single collection, and were considered non-exposed to PEDV. One-hundred serum samples were collected from five Italian farms in 2015. Samples were collected from nursery and grow-finish pigs.

Field samples with known PRCV or TGEV exposure. Forty four serum samples from nursery pigs without PEDV exposure were collected from four US farms in 2013; samples were positive for anti-PRCV (2 farms, $n = 24$) or anti-TGEV (2 farms, $n = 20$) antibodies by ELISA (Svanovir TGEV/PRCV-Ab, Boehringer Ingelheim Svanova, Uppsala, Sweden) (Gerber et al., 2014a).

Unknown exposure status

A total of 353 porcine serum samples originated from the US or Italy were used. Samples were arbitrarily selected and originated from 2014 pig case submissions to IZSLER (26 farms, $n = 151$) or to the ISU-VDL (36 farms, $n = 202$ which included 14 nursery pigs, 17 grow-finish pigs, 36 adult pigs and 135 pigs with unknown age) (Thachil et al., 2015).

2.2. PEDV antibody detection assays

A summary of the five assays used is presented in Table 1. All ELISAs were performed by personnel blinded to sample classification and without knowledge of the results obtained with any of the other ELISAs used in this comparison. All samples that originated from previous studies were (re)-tested with all five assays for the present study to account for any storage effect.

ELISA 1. The commercial G2b-PEDV N protein (NP)-based Swinecheck[®] PED indirect ELISA (Biovvet, Quebec, Canada) was used according to the manufacturer's label instructions. The S/P ratios ≥ 0.4 were considered antibody positive and < 0.4 as negative.

ELISA 2. The G2b-PEDV NP-based ID Screen[®] PEDV Indirect Screening test (IDvet, Montpellier, France) was used according to the manufacturer's label instructions. An S/P % ratio $\geq 60\%$ was considered antibody positive and $< 60\%$ was considered negative.

ELISA 3. An *in-house* G2b-PEDV S1-based indirect ELISA was used as previously described (Gerber et al., 2014a). The S/P ratios of > 0.2 were considered antibody positive,

between 0.14-0.2 as suspect and <0.14 as negative. Samples classified in the suspect range of the ELISA-3 were considered positive for analysis purposes.

ELISA 4. The *in-house* G2b-PEDV NP-based ELISA ID Screen[®] PEDV Competition 2.0 (IDvet, Montpellier, France) which is not commercially available was used. In brief, serum samples, diluted 1:2.5, were added to a G2b-PEDV NP protein coated 96-well plate for 2 h at 37°C. After a washing step, a peroxidase-conjugated anti-PEDV NP protein antibody was added and incubated at 37°C for 30 min. The peroxidase reaction was visualized by using tetramethylbenzidine-hydrogen peroxide solution as the substrate for 15 min at room temperature and stopped by adding 100 µL of 0.5 M sulfuric acid to each well. Optical densities (OD) were measured at 450 nm using an ELISA plate reader. An S/N % ratios $\leq 60\%$ was considered antibody positive and $> 60\%$ was considered negative.

ELISA 5. An *in-house* G1a PEDV WV ELISA was used. In brief, a G1a PEDV strain CV777-based WV ELISA was developed and validated at IZSLER based on a double antibody sandwich previously described (Sozzi et al., 2010). The ELISA microplates were coated with the 1F12 capture monoclonal antibody (MAb) (Sozzi et al., 2010). Serum samples, diluted 1:4, were mixed with equal volumes of whole cell-cultured adapted PEDV strain CV777 which was inactivated with β -propiolactone to conserve antigenic properties and pre-incubated in an auxiliary microplate for 1 h at 37°C. Then, 50 µl of the pre-incubated mixtures were transferred into a 1F12 MAb-coated plate and the conjugated horseradish peroxidase MAb 4C3 (Sozzi et al., 2010) was added. Following a further 1h incubation at 37°C the plate was washed. The optimal antigen concentration of the inactivated WV ELISA to obtain an optical density value of 1.0-1.5 in absence of a testing sample has been previously determined by testing serial two-fold dilutions of the cell supernatants. The colorimetric reaction was performed and optical densities (OD) were measured at 492 nm using an ELISA plate reader. Results were calculated by determining the absorbance value reduction, expressed as percentage of inhibition (PI)

having the control wells as reference. The antibody-blocking reaction was considered positive if the PI was $\geq 60\%$.

2.3. Discrepant results

Samples with discrepant results, defined as a sample that exhibited a positive result in one (or more) assay(s) and a negative result in another assay, were retested with all assays. For a given assay, discordant samples were tested two or three times and the final result was based on two coincident results independent of the retest results with any other assay. This approach was adopted according to standard practices in Veterinary Diagnostic Laboratories for samples with unexpected results. Initially, 153 samples were classified as discrepant. From those 138 remained discrepant after retesting and 15 samples were reclassified. Samples that were positive by only one assay after retesting were submitted to the ISU-VDL for PEDV IFA-ISU (Thomas et al., 2015) and for the PEDV WV ELISA (abbreviated here as WV-ISU) (Thomas et al., 2015) in addition to submission to the DTU National Veterinary Laboratory in Denmark for a blocking ELISA (abbreviated here as DTU-bELISA) (Lohse et al., 2016).

2.4. Statistical analysis

Experimental samples were excluded from the statistical analysis. The sensitivity and specificity of the assays were estimated using the field samples with known or unknown PEDV infection status collected at a single time point by a Bayesian latent class model with Markov Chain Monte Carlo (MCMC) simulation. A five test, three population, conditional independence model was fitted using JAGS 4.2.0. For samples with known infection status, the test outcomes were modeled by Bernoulli random variables with “chance of positive=sensitivity” for positive infection status and “1-specificity” for negative infection status. For samples with unknown infection status, latent Bernoulli random variables were first

generated for the infection status, and then the test outcomes were generated conditional on the latent status. Non-informative prior Beta distributions (0.5,0.5) were used for the parameters in the Bayesian model, ie, sensitivities, specificities and prevalence. The simulation was conducted with 1 chain with 20,000 iterations and the first 10,000 iterations were discarded as burning stage. The Gelman and Rubin diagnostic was used to check the convergence of three MCMC chains run in parallel.

The Cochran's Q test for matched data, followed by McNemar's test for pairwise comparisons was used to determine whether the proportions of positive samples were significantly different by assays. Differences among tests were considered significant if $p < 0.05$. For field samples, analysis kappa (κ) index was performed to determine the agreement of positive/negative results between assays. The strength of agreement was considered as described ≤ 0 = poor, 0.01–0.2 = slight, 0.21–0.4 = fair, 0.41–0.60 = moderate, 0.61–0.80 = substantial, and 0.81–1 = almost perfect. Statistical analyses were performed using SAS Version 9.4 (SAS Institute, Inc., Cary, USA).

3. Results

3.1. PEDV antibody detection in experimentally infected pigs

Results from experimentally infected pigs are summarized in Table 2. All 22 samples from PEDV unexposed pigs were negative by all assays. All five assays correctly identified at least one pig in each group of 3 to 6 pigs experimentally infected with G1a PEDV, G1b PEDV or G2b PEDV.

Among the pigs infected with G1a PEDV, positive pigs were first recognized by ELISA 3 at dpi 10, by ELISAs 1 and 4 at dpi 14 and by ELISAs 2 and 4 at dpi 28 (Table 2). Among the pigs infected with G1b PEDV, ELISAs 2 and 5 detected positive pigs by dpi 7. Among the pigs infected with the G2b-PEDV, all assays but ELISA 1 detected at least one positive pig at

dpi 7. After dpi 14 all assays had similar detection. Positive detection among the assays for a given dpi was not different although ELISA 1 presented the overall lowest numerical values.

3.2. *PEDV antibody detection in field serum samples*

Field samples with known PEDV exposure. All assays identified at least one positive sample in all of the 13 Italian PEDV exposed farms. In the 10 farms from which samples were collected during the G1b PEDV outbreaks in 2014/2015 the positive detection varied from 95-100%. In the three farms from which samples were collected during the G1a PEDV outbreaks in 2006, the positive detection varied from 32 to 96% among assays (Table 3). Specifically, ELISA 5 detected 96.0% (24/25) positive samples from all three farms, ELISAs 2, 3 and 4 detected 40.0% (10/25) to 52.0% (13/25) samples as positive and ELISA 1 detected 32.0% (8/25) samples as positive.

3.2.1 Field samples without PEDV exposure. From the 100 samples originated from the five 2014/2015 Italian farms without known PEDV exposure, ELISA 3 identified 16% (Table 4, Farm 6) of the samples as positive, ELISA 1 identified 6% (Farms 6, 5 and 7) positive samples and ELISA 2 (Farm 7) identified 1% positive samples. The positive sample detected by ELISA 2 on Farm 7 was also detected as positive by ELISA 1. The other ELISAs did not detect any positive sample in the PEDV negative samples set.

3.2.2 Field samples with unknown PEDV exposure. ELISA 5 classified the highest number of positive samples collected in 2014/2015 from 26 Italian farms with unknown exposure ($p < 0.05$) (Table 3). Specifically, ELISA 5 identified 88.4% (23/26) of the farms as PEDV positive, while ELISAs 1, 2, 3 and 4 classified 30.7% (8/26) to 50% (13/26) of the farms as PEDV positive. ELISA 3 classified the highest number of positive samples within the 36 US farms with unknown exposure ($p < 0.05$) (Table 3). ELISAs 1, 3, 4 and 5 classified 61.1%

(22/36) to 77.7% (28/36) of the farms as positive and ELISA 2 classified 44.4% (16/36) of the farms as positive.

3.2.3 Overall agreement on all field serum samples regardless of PEDV exposure status. ELISA 5 presented the overall highest number of positive samples (48%, 315/647) and ELISAs 1 (28%, 175/622) and 4 (29%, 178/622) presented the lowest detection (Table 3). There was an overall substantial agreement ($\kappa = 0.61$) among assays. The agreement among assays was similar considering the sample set origin (Italy, $\kappa = 0.61$; US, $\kappa = 0.56$). Interestingly, the agreement among assays in the subset of samples originating from farms with known exposure to PEDV ($n = 269$) was substantial (overall agreement $\kappa = 0.80$, $\kappa = 0.71$ - 0.95), while the agreement among assays on samples of unknown status ($n = 353$) was only fair (overall agreement $\kappa = 0.31$, $\kappa = 0.16$ - 0.62).

3.3. Cross-reaction with other coronaviruses

Among 44 TGEV or PRCV antibody positive serum samples, 1/24 anti-PRCV antibody positive sample and 2/20 anti-TGEV antibody positive samples reacted with ELISA 2 (Table 3). Positive samples originated from Farm A, one of the two PRCV infected farms, and from Farm B, one of two TGEV infected farms (Table 4). ELISA 5 reacted with one TGEV antibody positive sample originating from Farm C. Positive signals on any of the PRCV or TGEV samples were not observed with the other ELISAs.

3.4. Samples with discrepant results

Samples with discrepant results ($n = 138$), defined as a sample that did not exhibit coincidental results with all assays, i.e., exhibited positive results in one or more assays and negative results in any of the other assays, were further evaluated based on sample availability. A total of 96 samples from 28 farms were selected and tested by an IFA and the WV-ISU

ELISA, and 83 of the 96 samples were tested by the DTU-bELISA (Table 4). The WV-ISU ELISA classified the highest number of farms as positive (23/26), including a sample on a PRCV positive, PEDV negative farm, while the IFA classified the lowest number of farms as positive (4/28). The DTU-bELISA classified 7/28 farms as positive.

3.5. Estimate of diagnostic sensitivity and specificity

Assays accuracy were estimated by using cumulative data using a Markov Chain Monte Carlo simulation. The estimate of expected predictive error (DIC) was 2947.5. Diagnostic sensitivity and specificity with a 95% probability interval were estimated to be 68.2% (62.1 – 74.4%) and 97.5% (95.2 – 99.0%) for ELISA 1, 73.7% (71.5 – 79.6%) and 98.4% (96.6 – 99.5%) for ELISA 2, 86.2% (81.1 – 90.6%) and 91.6% (87.7 – 94.8%) for ELISA 3, 78.3% (72.8 – 83.5%) and 99.7% (98.2 – 100%) for ELISA 4, and 93.5% (90.3 – 96.0%) and 91.2% (83.8 – 97.9%) for ELISA 5.

4. Discussion

The ongoing large-scale PEDV outbreaks in the Americas, Asia and Europe demand specific PEDV detection tools such as serology for locating PEDV-exposed herds, to conduct epidemiological studies, and to do immunization efficacy studies (Lin et al., 2015). Challenges for accurate serological diagnosis for PEDV include the variability of viral genome and also the existence of common immunodominant sites among alphacoronaviruses (Lin et al., 2015). This includes TGEV that produces similar clinical and pathological signs as PEDV (Stevenson et al., 2013) and PRCV that is widespread in many geographic locations (Saif et al., 2012). In this study, the ability to detect antibodies against PEDV in pigs infected with G1 or G2 PEDV isolates was compared for five serological tests based on PEDV WV, the N protein or the S protein.

All five tested assays correctly identified pigs infected with G1a PEDV, G1b PEDV or G2b PEDV but with different detection proportions. None of the assays had a consistently higher detection across the different strains. It has been suggested that ELISAs based on WV or the N protein would be more sensitive for detection of antibodies against heterologous PEDV strains than ELISAs based on the S1 protein (Chen et al., 2016b; Lin et al., 2015). This could not be confirmed in the present study as by using the S1-based ELISA 3 pigs infected with G1a PEDV were identified up to four days earlier compared to all other assays, including ELISA 5 that is based on the whole virus of a G1a PEDV strain. ELISA 5 and N-based ELISA 4 detected antibodies against G1b PEDV earlier compared to the S1-based ELISA 3. However, the latter had similar performance in the detection of the G1b PEDV sera compared to the other two N-based ELISAs indicating that other factors than the antigen used could contribute to differences in the sensitivity among assays.

In the present study, the NP-based indirect ELISA 2 cross-reacted with 1/24 PRCV and 2/20 TGEV positive serum samples, and the WV-based competitive ELISA 5 cross-reacted with a TGEV positive serum sample. Indirect ELISAs have the advantage of requiring only one anti-species specific conjugate targeting a particular antibody subset such as anti IgA or IgG that are readily available, making it suitable for wide use for screening large number of samples (Schrijver and Kramps, 1998). However, in indirect ELISAs, non-specific binding of antibodies is a common problem, because all antibodies that are bound in the well, either specifically or non-specifically, will be detected by the conjugated antibody, affecting its specificity (Schrijver and Kramps, 1998). On the other hand, competitive ELISAs are generally recognized as being more specific than indirect ELISAs because interference due to antibody non-specific binding generally does not bias the test result and allow the use of partially purified virus when highly purified MAbs are used (Schrijver and Kramps, 1998). Because of this characteristic, serum samples can be tested in low dilutions, which may contribute also to

a higher sensitivity of this format (OIE, 2015). However, unexpected interferences can also occur in competitive ELISAs. For example, purified WV contains all virally expressed proteins, which increases sensitivity but also increases the risk for cross-reaction with similar viruses (Simmons, 2008). Truncation of the antigenic site in the N protein N-terminal region to exclude the immunodominant cross-reactive epitope or the usage of S protein as antigen for PEDV serology has been suggested to improve specificity of the immunoassays (Lin et al., 2015).

ELISA 3 detected 16/34 samples from a PEDV non-exposed farm as positive. Another NP-based assay (ELISA 1) also detected a single positive sample on the same farm. When the discrepant samples were further evaluated using an IFA, the indirect WV-ISU ELISA, the blocking DTU-bELISA contradictory results of these additional assays further complicated the serological diagnosis. As the 2015 non-exposed Italian farms were classified based on absence of clinical signs and PEDV RT-PCR negative results in fecal samples, the result suggests an unspecific reaction by one of the assays used. However, the high number of samples detected in a single farm (Farm 6) by ELISA 3 could indicate a cross-reaction with an unknown pathogen or a true positive result. Alignment of the S1 gene sequence and sequences publicly available through GenBank did not reveal similarities with any other pig pathogens (data not shown). Alternatively, the farm could have experienced a prior subclinical PEDV infection as the absence of PEDV RNA was based on a single point RT-PCR testing on ten fecal samples. Misclassifications of farms as “negative” could lead to artificially decreased specificity of assays. This bias in the specificity usually occurs because the positive serological results within these farms are classified as false-positives. As the farms classified as negative were excluded from the sensitivity analysis, this did not affect the estimated sensitivity. Previous studies have shown that PEDV antibodies in serum samples can be detected up to 7 months after initial exposure (Goede et al., 2015; Ouyang et al., 2015). Basing a negative farm classification on

testing fecal samples by RT-PCR at a single time point could easily lead to a misclassification if the farm had experienced a clinically not recognized PEDV infection weeks or months before the testing. Phylogenetic studies showed that the PEDV strains currently associated with PEDV outbreaks in Italy are G1b strains (Boniotti et al., 2016), which have been suggested to produce milder clinical signs when compared to G2b strains (Chen et al., 2016a).

Although the evaluated assays presented an overall substantial agreement for the field samples from both tested countries, the agreement differed within subsets. The almost perfect agreement on the samples with known exposure may be explained by relative timing of PEDV infection (acute to subacute outbreaks) to sample collection and therefore the high number of positive samples, which could have artificially increased the estimates of sensitivity of the assays. In the unknown exposure set, samples likely contained animals in different phase of infection and samples with variable amounts of antibodies. Additionally, the unknown infection status for PEDV and other possible coronaviruses infections complicate the interpretation of discrepant results. ELISA 5, developed in Italy, had a much higher detection of positive serum samples in an Italian sample set with unknown PEDV exposure but not for the US subset of unknown samples. An increased positive detection by the competitive ELISA may be due to detection of IgA and IgM antibodies in addition to the IgG antibodies detected by the indirect ELISAs. However, the same trend was not observed with the competitive ELISA 4. Similarly, ELISA 3, developed in the US, had the highest detection among the US unknown samples subset. The sample set used for each assay optimization could partially explain the different detection proportions (Banoo et al., 2010) as the usage of same cut off for geographically different animal populations infected with different viral strains may result in different sensitivity and specificity. Similarly, the usage of only experimentally infected animals can lead to inflated estimates of accuracy when the samples have been collected only from animals at the peak of antibody production, or from animals from herds tested free for the

most pathogens (Banoo et al., 2010). Alternatively, a PEDV-TGEV recombinant virus was found to circulate in Italy during 2009-2012 (Boniotti et al., 2016). Although the PEDV-TGEV recombinant virus has not been detected after 2012 (Boniotti et al., 2016), the presence of unidentified recombinant enteric coronaviruses could perhaps partially explain a higher positive detection for the WV-based ELISA 5.

Ideally, diagnostic specificity and sensitivity should be calculated using unequivocally true negative and true positive samples as determined by a combination of ‘gold standard’ methods (OIE, 2015). However, in the absence of ‘gold standard’ tests, latent-class models based on reference populations, and not individual reference samples can be used (OIE, 2015). In the present study, a Bayesian approach was used to estimate the assays accuracy. Measures of diagnostic accuracy are extremely sensitive to the design of the study and although the usage of samples from different geographical populations herein could potentially increase representativeness, the accuracy of the presented results should be interpreted with caution. When applying a diagnostic assay, laboratories should observe if the cut-off values are applicable in their specific context and revise diagnostic sensitivity and specificity as required (Crowther et al., 2006). It is critical that the diagnostic purpose of an assay is clearly defined in regard to the population to be tested (OIE, 2015). As diagnostic sensitivity and diagnostic specificity are usually inversely related and as such a decrease in diagnostic specificity will result in elevated false positive results. Tests with moderate to high diagnostic sensitivity and specificity balance the false positive and false negative results and give a more accurate estimate of prevalence of infection or vaccination within a population (OIE, 2015). The decision which assay should be used for PEDV antibodies monitoring at the herd level ultimately needs to be based on the expected prevalence of disease, desired specificity and sensitivity levels (screening or confirmatory test), easiness of testing, and access to a certain assay.

5. Conclusions

In summary, the ELISA 1 had the overall lowest detection from sample subsets derived from both experimentally and naturally infected animals and ELISA 5 had the overall highest detection.

Financial support

Funding for this study was provided by the Biotechnology and Biological Sciences Research Council (BBSRC) Institute Strategic Programme Grant awarded to the Roslin Institute (BB/J004324/1; BBS/E/D/20241864). ID Screen[®] PEDV Indirect Screening test and ID Screen[®] PEDV Competition 2.0 ELISA kits were provided by IDVet, Grables, France.

Conflict of interest

The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Authors L. Comtet, M. Roche and P. Pourquier were employed by IDVet Innovative Diagnostics when this study was conducted. The remaining authors declare no conflicting interests.

Acknowledgments

We thank Mark McNeil for assistance with the sample testing.

References

Banoo, S., Bell, D., Bossuyt, P., Herring, A., Mabey, D., Poole, F., Smith, P.G., Sriram, N., Wongsrichanalai, C., Linke, R., O'Brien, R., Perkins, M., Cunningham, J., Matsoso, P., Nathanson, C.M., Olliaro, P., Peeling, R.W., Ramsay, A., 2010. Evaluation of diagnostic tests for infectious diseases: general principles. *Nat. Rev. Microbiol.* 8, S17-S29.

- Boniotti, M.B., Papetti, A., Lavazza, A., Alborali, G., Sozzi, E., Chiapponi, C., Faccini, S., Bonilauri, P., Cordioli, P., Marthaler, D., 2016. Porcine Epidemic Diarrhea Virus and Discovery of a Recombinant Swine Enteric Coronavirus, Italy. *Emerg. Infect. Dis.* 22, 83-87.
- Brian, D.A., Baric, R.S., 2005. Coronavirus genome structure and replication. *Curr. Top. Microbiol Immunol* 287, 1-30.
- Bridgen, A., Duarte, M., Tobler, K., Laude, H., Ackermann, M., 1993. Sequence determination of the nucleocapsid protein gene of the porcine epidemic diarrhoea virus confirms that this virus is a coronavirus related to human coronavirus 229E and porcine transmissible gastroenteritis virus. *J Gen Virol* 74 (Pt 9), 1795-1804.
- Chen, Q., Gauger, P.C., Stafne, M.R., Thomas, J.T., Madson, D.M., Huang, H., Zheng, Y., Li, G., Zhang, J., 2016a. Pathogenesis comparison between the United States porcine epidemic diarrhea virus prototype and S-INDEL-variant strains in conventional neonatal piglets. *J. Gen. Virol.*
- Chen, Q., Li, G., Stasko, J., Thomas, J.T., Stensland, W.R., Pillatzki, A.E., Gauger, P.C., Schwartz, K.J., Madson, D., Yoon, K.J., Stevenson, G.W., Burrough, E.R., Harmon, K.M., Main, R.G., Zhang, J., 2014. Isolation and characterization of porcine epidemic diarrhea viruses associated with the 2013 disease outbreak among swine in the United States. *J. Clin. Microbiol.* 52, 234-243.
- Chen, Q., Thomas, J.T., Gimenez-Lirola, L.G., Hardham, J.M., Gao, Q., Gerber, P.F., Opriessnig, T., Zheng, Y., Li, G., Gauger, P.C., Madson, D.M., Magstadt, D.R., Zhang, J., 2016b. Evaluation of serological cross-reactivity and cross-neutralization between the United States porcine epidemic diarrhea virus prototype and S-INDEL-variant strains. *BMC. Vet. Res.* 12, 70.
- Crowther, J.R., Unger, H., Viljoen, G.J., 2006. Aspects of kit validation for tests used for the diagnosis and surveillance of livestock diseases: producer and end-user responsibilities. *Rev. Sci. Tech.* 25, 913-935.
- Gerber, P.F., Gong, Q., Huang, Y.W., Wang, C., Holtkamp, D., Opriessnig, T., 2014a. Detection of antibodies against porcine epidemic diarrhea virus in serum and colostrum by indirect ELISA. *Vet. J.* 202, 33-26.
- Gerber, P.F., Xiao, C.T., Chen, Q., Zhang, J., Halbur, P.G., Opriessnig, T., 2014b. The spray-drying process is sufficient to inactivate infectious porcine epidemic diarrhea virus in plasma. *Vet. Microbiol.* 174, 86-92.
- Goede, D., Murtaugh, M.P., Nerem, J., Yeske, P., Rossow, K., Morrison, R., 2015. Previous infection of sows with a "mild" strain of porcine epidemic diarrhea virus confers protection against infection with a "severe" strain. *Vet. Microbiol.* 176, 161-164.
- Hofmann, M., Wyler, R., 1990. Enzyme-linked immunosorbent assay for the detection of porcine epidemic diarrhea coronavirus antibodies in swine sera. *Vet Microbiol* 21, 263-273.
- Hou, X.L., Yu, L.Y., Liu, J., 2007. Development and evaluation of enzyme-linked immunosorbent assay based on recombinant nucleocapsid protein for detection of porcine epidemic diarrhea (PEDV) antibodies. *Vet Microbiol* 123, 86-92.

- Huang, Y.W., Dickerman, A.W., Pineyro, P., Li, L., Fang, L., Kiehne, R., Opriessnig, T., Meng, X.J., 2013. Origin, evolution, and genotyping of emergent porcine epidemic diarrhea virus strains in the United States. *MBio*. 4, e00737-13.
- Knuchel, M., Ackermann, M., Muller, H.K., Kihm, U., 1992. An ELISA for detection of antibodies against porcine epidemic diarrhoea virus (PEDV) based on the specific solubility of the viral surface glycoprotein. *Vet Microbiol* 32, 117-134.
- Lee, C., 2015. Porcine epidemic diarrhea virus: An emerging and re-emerging epizootic swine virus. *Viol. J.* 12, 193.
- Lin, C.M., Gao, X., Oka, T., Vlasova, A.N., Esseili, M.A., Wang, Q., Saif, L.J., 2015. Antigenic relationships among porcine epidemic diarrhea virus and transmissible gastroenteritis virus strains. *J. Virol.* 89, 3332-3342.
- Lohse, L., Krog, J.S., Strandbygaard, B., Rasmussen, T.B., Kjaer, J., Belsham, G.J., Botner, A., 2016. Experimental Infection of Young Pigs with an Early European Strain of Porcine Epidemic Diarrhoea Virus and a Recent US Strain. *Transbound. Emerg. Dis.*
- Martelli, P., Lavazza, A., Nigrelli, A.D., Merialdi, G., Alborali, L.G., Pensaert, M.B., 2008. Epidemic of diarrhoea caused by porcine epidemic diarrhoea virus in Italy. *Vet. Rec.* 162, 307-310.
- Masters, P.S., 2006. The molecular biology of coronaviruses. *Adv Virus Res* 66, 193-292.
- Oh, J.S., Song, D.S., Yang, J.S., Song, J.Y., Moon, H.J., Kim, T.Y., Park, B.K., 2005. Comparison of an enzyme-linked immunosorbent assay with serum neutralization test for serodiagnosis of porcine epidemic diarrhea virus infection. *J Vet Sci* 6, 349-352.
- OIE, 2015. Manual of standards for diagnostic tests and vaccines for terrestrial animals.
- Okda, F., Liu, X., Singrey, A., Clement, T., Nelson, J., Christopher-Hennings, J., Nelson, E.A., Lawson, S., 2015. Development of an indirect ELISA, blocking ELISA, fluorescent microsphere immunoassay and fluorescent focus neutralization assay for serologic evaluation of exposure to North American strains of Porcine Epidemic Diarrhea Virus. *BMC. Vet. Res.* 11, 180.
- Opriessnig, T., Xiao, C.T., Gerber, P.F., Zhang, J., Halbur, P.G., 2014. Porcine epidemic diarrhea virus RNA present in commercial spray-dried porcine plasma is not infectious to naive pigs. *PLoS. One.* 9, e104766.
- Ouyang, K., Shyu, D.L., Dhakal, S., Hiremath, J., Binjawadagi, B., Lakshmanappa, Y.S., Guo, R., Ransburgh, R., Bondra, K.M., Gauger, P., Zhang, J., Specht, T., Gilbertie, A., Minton, W., Fang, Y., Renukaradhya, G.J., 2015. Evaluation of humoral immune status in porcine epidemic diarrhea virus (PEDV) infected sows under field conditions. *Vet. Res.* 46, 140.
- Saif, L.J., Pensaert, M., Sestak, K., Yeo, S.G., Jung, K., 2012. Coronaviruses. In: Zimmerman, J.J., Karriker, L., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), *Diseases of Swine*. Wiley-Blackwell, Ames, pp. 501-524.
- Schrijver, R.S., Kramps, J.A., 1998. Critical factors affecting the diagnostic reliability of enzyme-linked immunosorbent assay formats. *Rev. Sci. Tech.* 17, 550-561.

- Simmons, J.H., 2008. Development, application, and quality control of serology assays used for diagnostic monitoring of laboratory nonhuman primates. *ILAR. J.* 49, 157-169.
- Song, D., Moon, H., Kang, B., 2015. Porcine epidemic diarrhea: a review of current epidemiology and available vaccines. *Clin. Exp. Vaccine Res.* 4, 166-176.
- Song, D., Park, B., 2012. Porcine epidemic diarrhoea virus: a comprehensive review of molecular epidemiology, diagnosis, and vaccines. *Virus Genes* 44, 167-175.
- Sozzi, E., Luppi, A., Lelli, D., Martin, A.M., Canelli, E., Brocchi, E., Lavazza, A., Cordioli, P., 2010. Comparison of enzyme-linked immunosorbent assay and RT-PCR for the detection of porcine epidemic diarrhoea virus. *Res Vet Sci* 88, 166-168.
- Stevenson, G.W., Hoang, H., Schwartz, K.J., Burrough, E.R., Sun, D., Madson, D., Cooper, V.L., Pillatzki, A., Gauger, P., Schmitt, B.J., Koster, L.G., Killian, M.L., Yoon, K.J., 2013. Emergence of Porcine epidemic diarrhea virus in the United States: clinical signs, lesions, and viral genomic sequences. *J Vet Diagn Invest* 25, 649-654.
- Thachil, A., Gerber, P.F., Xiao, C.T., Huang, Y.W., Opriessnig, T., 2015. Development and application of an ELISA for the detection of porcine deltacoronavirus IgG antibodies. *PLoS. One.* 10, e0124363.
- Thomas, J.T., Chen, Q., Gauger, P.C., Gimenez-Lirola, L.G., Sinha, A., Harmon, K.M., Madson, D.M., Burrough, E.R., Magstadt, D.R., Salzbrenner, H.M., Welch, M.W., Yoon, K.J., Zimmerman, J.J., Zhang, J., 2015. Effect of Porcine Epidemic Diarrhea Virus Infectious Doses on Infection Outcomes in Naive Conventional Neonatal and Weaned Pigs. *PLoS. One.* 10, e0139266.

Table 1

Summary of the enzyme-linked immunosorbent assays used in this study

Assay	Test principle	Coating antigen	PEDV genogroup (G)	Serum dilution	Conjugate	Total incubation time (h:m)	Reference
ELISA 1	Indirect	Nucleoprotein	G2b	1:200	Anti-porcine IgG	03:10	Swinecheck PED indirect, Biovet IDScreen
ELISA 2	Indirect	Nucleoprotein	G2b	1:10	Protein A/G	01:30	PEDV Indirect, IDvet
ELISA 3	Indirect	Spike 1	G2b	1:100	Anti-porcine IgG	01:10	Gerber et al., 2014
ELISA 4	Competitive	Nucleoprotein	G2b	1:2.5	Anti-PEDV nucleoprotein antibody	02:45	This study
ELISA 5	Competitive	Whole virus	G1a	1:2/1:4	Monoclonal antibody anti-G1a PEDV	02:15	This study

Table 2

Detection rates of anti-PEDV antibodies in serum samples of pigs experimentally infected with PEDV genogroup 1a (G1a) strain Br-1/87, PEDV G1b strain USA/IL/2014/20697, or PEDV genogroup 2b (G2b) US prototype strain ISU13-19338E at various days post infection tested by five different assays (ELISA 1, ELISA 2, ELISA 3, ELISA 4, ELISA 5). Values in parenthesis were classified as suspect.

Assay	Days post infection											
	G1a					G1b				G2b		
	10	14	17	21	28	7	14	21	28	7	14	21
ELISA 1	0/3	2/3	2/3	2/3	2/3	0/6	2/6	3/6	2/6	0/6	4/6	10/13
ELISA 2	0/3	0/3	0/3	0/3	2/3	2/6	5/6	5/6	5/6	1/6	4/6	13/13
ELISA 3	1/3	3/3	3/3	3/3	3/3	0/6	3(2)/6	4(1)/6	4/6	3/6	6/6	13/13
ELISA 4	0/3	0/3	0/3	0/3	2/3	0/6	2/6	6/6	6/6	2/6	4/6	13/13
ELISA 5	0/3	1/3	1/3	1/3	1/3	4/6	5/6	5/6	5/6	3/6	6/6	13/13

Table 3

Detection rates of anti-PEDV antibodies in field serum samples tested by five different ELISAs (1 through 5). Samples were collected from pigs originating from Italian or US commercial farms with known or unknown PEDV exposure as determined by RT-PCR on fecal samples.

Country	PEDV exposure (Genogroup)	Year	TGEV/PRCV exposure	N. samples	N. positive samples per assay				
					ELISA 1	ELISA 2	ELISA 3	ELISA 4	ELISA 5
Italy	Exposed (G1a)	2006	Unknown	25	10 ^b	13 ^{ab}	13 ^{ab}	8 ^b	24 ^a
	Exposed (G1b)	2015	Unknown	100	95	94	98	98	100
	Non-exposed	2015	Unknown	100	5	1	16	0	0
	Unknown	2014/2015	Unknown	151	10 ^c	12 ^c	21 ^b	21 ^b	108 ^a
US	Non-exposed	2013	Exposed	44	0	3	0	0	1
	Unknown	2014	Unknown	202	37 ^c	59 ^b	69 ^a	30 ^c	55 ^b
Total				622	175^d	211^c	229^b	178^d	315^a

* Different superscripts (a, b, c) indicate difference of detection rates among kits for a given sample origin (McNemar's test, $p < 0.05$).

Table 4

Detection rates of anti-PEDV antibodies in field serum samples ($n = 96$) with discordant results, defined as a sample that exhibited a positive result in one or more ELISA and a negative result in another ELISA(s). Discordant samples were additionally tested by IFA, WV-ISU ELISA and the DTU-bELISA. Results were presented as number of positive tested samples for a given assay per farm. Light grey indicates that only one tested sample was positive with a given test and dark grey indicates that more than one sample was positive for a given test.

Country	Year	Farm	PEDV status	ELISAs used in this study					Confirmatory assays		
				ELISA 1	ELISA 2	ELISA 3	ELISA 4	ELISA 5	IFA	WV-ISU	DTU-bELISA
Italy	2014	1	Unknown	0/8	0/8	0/8	0/8	4/8	0/4	4/4	0/3
Italy	2014	2	Unknown	0/1	0/1	0/1	0/1	1/1	1/1	1/1	1/1
Italy	2014	3	Unknown	0/6	0/6	0/6	0/6	5/6	0/4	2/4	0/4
Italy	2014	4	Unknown	0/8	0/8	0/8	0/8	7/8	0/7	3/7	3/7
Italy	2014	8	Unknown	1/4	0/4	0/4	0/4	4/4	0/3	3/3	0/3
Italy	2014	9	Unknown	0/6	0/6	0/6	1/6	2/6	0/1	0/1	0/1
Italy	2014	10	Unknown	0/6	0/6	0/6	2/6	3/6	0/3	3/3	0/2
Italy	2014	11	Unknown	0/8	0/8	0/8	4/8	5/8	0/3	3/3	1/2
Italy	2014	12	Unknown	1/4	0/4	0/4	0/4	4/4	0/3	3/3	0/3
Italy	2014	13	Unknown	0/2	0/2	0/2	1/2	2/2	0/1	0/1	0/1
Italy	2014	14	Unknown	0/3	0/3	2/3	0/3	1/3	0/1	1/1	1/1
Italy	2014	15	Unknown	2/4	0/4	0/4	0/4	3/4	0/1	1/1	0/1
Italy	2014	16	Unknown	0/12	0/12	0/12	5/12	7/12	1/6	3/6	0/6
Italy	2014	17	Unknown	0/3	1/3	0/3	1/3	3/3	0/2	2/2	2/2
Italy	2014	18	Unknown	2/10	1/10	0/10	0/10	7/10	0/6	6/6	0/6
Italy	2014	19	Unknown	1/4	1/4	2/4	1/4	4/4	0/3	2/2	0/1
Italy	2014	20	Unknown	1/10	1/10	1/10	3/10	6/10	0/4	2/4	0/4
Italy	2014	21	Unknown	1/6	2/6	0/6	2/6	3/6	0/3	3/3	0/1
Italy	2014	22	Unknown	1/10	0/10	1/10	1/10	5/10	0/5	5/5	0/4
Italy	2014	23	Unknown	0/9	1/9	2/9	0/9	8/9	0/4	2/4	0/4
Italy	2014	25	Unknown	1/17	5/17	4/17	2/17	16/17	0/7	7/7	1/7
Italy	2015	5	Non-exposed	2/18	0/18	0/18	0/18	0/18	1/2	1/2	0/2
Italy	2015	6	Non-exposed	1/34	0/34	16/34	0/34	0/34	0/13	10/13	0/12
Italy	2015	7	Non-exposed	3/20	1/20	0/20	0/20	0/20	0/2	2/2	0/22
US	2013	A	Non-exposed	0/6	1/6	6/6	0/6	0/6	5/5	1/1	0/3
US	2013	B	Non-exposed	0/10	2/10	0/10	0/10	0/10	0/2	nt	nt
US	2013	C	Non-exposed	0/10	0/10	0/10	0/10	1/10	0/2	nt	nt
Italy	2015	24	Exposed	10/11	10/11	10/11	10/11	11/11	1/1	1/1	1/1

*nt = not tested.